

Effects of different O₂ concentrations on nitrogenase activity, respiration, and O₂ diffusion resistance in *Lupinus albus* L. cv. Multolupa nodules

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Summary

Nitrogenase, responsible for N₂ fixation in legume nodules, can be denatured by O₂ and functions under microaerobic conditions. However, O₂ is required for ATP production associated with nitrogenase activity; therefore, O₂ concentration within infected cells is highly regulated by a combination of nodule respiration, leghaemoglobin, and an O₂ diffusion barrier. To investigate O₂ diffusion regulation, different concentrations (1 %, 21 %, and 40 % (v/v)) were applied to nodulated root systems of *Lupinus albus* for 3 and 10 days.

The application of 1 % O₂ for 3 days produced a marked decrease in nitrogenase activity, up to 30 % of that of control (21 % O₂) plants. The irreversible failure of nitrogenase functioning occurred after 10 days and could not be explained by the small variations in the content of nitrogenase components I and II. The internal O₂ concentration seems to be a limiting factor to N₂ fixation, but nodules can partly maintain their activity if hypoxic conditions are not too prolonged.

The nitrogenase inhibition of the 3-day 40 % O₂ treatment was moderate (<20 %), and after 10 days nitrogenase activity returned to control values. Again, variations in nitrogenase components were not correlated with the activity measured by acetylene reduction in a flow-through system. Root respiration rates were unexpectedly reduced. These plants seemed to adapt to 40 % O₂ by varying the minimum resistance (R_{min}) of the O₂ diffusion barrier, which was almost doubled from the third day of treatment.

Key words: *Lupinus albus* – nitrogen fixation – nitrogenase components – nitrogenase functioning – oxygen diffusion resistance

Introduction

Legumes have developed a symbiosis with bacteria from the Rhizobiaceae family in such a way that the bacteroids are able to satisfy plant nitrogen requirements through the activity

of the nitrogenase enzyme system. This prokaryotic enzyme is denatured at 5 mmol O₂ · m⁻³ (Sheehy and Thornley 1988), but rhizobia species are strictly dependent on a respiratory energy conservation (Appleby 1984, Hennecke et al. 1994), and nitrogenase function also needs O₂ as an electron acceptor for ATP production. These scarcely compatible conditions led legumes to incorporate several mechanisms into

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the nodule in order to create a highly regulated microaerobic environment. Indeed, a drastic decline in O_2 concentration has been previously detected by microelectrodes in the layers of cells adjacent to the infected zone (Tjepkema and Yocum 1974, Witty et al. 1987).

Several O_2 regulation mechanisms have been proposed, involving both the nodule cortex and the infected (bacteroid containing) zone. The O_2 diffusion barrier, located in the inner and middle cortex, has been proposed as the main mechanism of O_2 regulation (Minchin 1997, Layzell 1998). The operation of this cortical barrier has been previously described in nodules of *Lupinus albus* L. (De Lorenzo et al. 1993, Iannetta et al. 1993, 1995). Leghaemoglobin (Lb) also has a regulatory function (Appleby 1984) since it delivers O_2 directly to the bacteroids and allows free O_2 concentration at a very low level throughout most of the infected cells. However, the O_2 saturation of Lb near the intercellular air spaces may make an important contribution to limiting O_2 diffusion (Thumfort et al. 1994). Finally, bacteroid and mitochondria respiration can also function as a sink for oxygen and regulate the O_2 diffusion flux (Bergersen 1997a, b).

Some groups (Parson and Day 1990, Dakora and Atkins 1990a, b, James et al. 1991, Atkins et al. 1993, Iannetta et al. 1995) have observed structural changes in the nodule cortex when growing plants under different O_2 concentrations. Changes have also been observed under stress conditions such as detopping, high temperature, darkening, nitrate or salt (De Lorenzo et al. 1993, Serraj et al. 1995, Fernández-Pascual et al. 1996a). In these conditions, a nitrogenase activity decrease was accompanied by an increase in the resistance to O_2 diffusion (Minchin et al. 1992, Iannetta et al. 1993, De Lorenzo et al. 1993, Hunt and Layzell 1993). These stress-induced decreases in nitrogenase activity can be at least partially overcome by increasing the external O_2 concentration, although changes in carbon metabolism appear to occur under water and temperature stress or when nitrate is applied (Lang et al. 1993, Díaz del Castillo et al. 1994, Kuzma and Layzell 1994).

At the level of the bacteroids themselves, only respiration appears to be involved in the protection of nitrogenase from O_2 damage. Indeed, the use of high-pressure freezing techniques has recently demonstrated that the bacteroidal and peribacteroidal membranes are intimately related, and therefore, Lb-transported O_2 can be directly released to the respiratory chain of the bacteroid membrane (De Felipe et al. 1997). Also, the high rate of Lb O_2 release (7 times faster than bacteroid respiration) does not appear to be a limiting or regulating factor (Appleby 1994).

Some studies of O_2 regulation and adaptation mechanisms have been made over long time periods of 15 to 65 days (Parsons and Day 1990, James et al. 1991, Dakora and Atkins 1990a, Arrese-Igor et al. 1993), whilst other workers have used much shorter times of 1–2 minutes to 2 hours (Serraj et al. 1995, Iannetta et al. 1995, Denison and Kinraide 1995). The experiments on nodule oxygen diffusion, initiated with ni-

trate applications (De Lorenzo et al. 1993, Iannetta et al. 1993), were continued with this work. The regulation and the adaptation mechanisms were studied by growing lupin plants under suboptimal (1% O_2 (v/v)) and superoptimal O_2 (40% O_2 (v/v)) concentrations for 3 and 10 days between reversible short-term changes in the variable diffusion barrier and irreversible long-term morphological adaptations. The resulting hypothesis of those previously mentioned works was that the decrease in nitrogenase activity produced by nitrate was caused by an increase in O_2 diffusion resistance, reducing the oxygen available to the infected zone. The aim of the present work was to determine if the reduction of O_2 concentration itself produces similar effects on nitrogen fixation to those of nitrate application, as well as to probe more deeply into the O_2 diffusion mechanism inside the nodule.

Material and Methods

Plant material

Seeds of white lupin (*Lupinus albus* L. cv. Multolupa) were surface sterilised using 0.1% (w/v) $HgCl_2$ for 5 minutes, followed by 10 washes with sterilized distilled water, sown in 1-liter pots filled with vermiculite, inoculated at sowing with *Bradyrhizobium* sp. (*Lupinus*) strain ISLU 16, and allowed to grow in a controlled growth chamber. Growth conditions were: 16-h photoperiod, a quantum irradiance of $500 \mu mol \cdot m^{-2} \cdot s^{-1}$, and day/night temperatures of 25/15 °C. Plants were watered with a nitrogen-free nutrient solution (Lang et al. 1993), and 3 weeks after sowing plants were transferred to a greenhouse. Supplementary 400 W Philips HLRG lights were used.

Oxygen treatments

1% (v/v) O_2 concentration (1.1 kPa) was used as the suboptimal concentration and 40% (v/v) O_2 as the superoptimal concentration (44.6 kPa), both containing 350 ppm of CO_2 . Atmospheric O_2 was used as the control (21% (v/v)) concentration (23.4 kPa), also containing 350 ppm of CO_2 . Root systems were sealed into the growth pots using a non-toxic, plastic sealing compound (QTY1, Proteus Brass Division, UK). These oxygen concentrations were applied to sealed root systems (Guasch 1998) at a flow rate of $100 mL \cdot min^{-1} \cdot plant^{-1}$ for 3 or 10 days (starting 39 and 32 days after sowing, respectively). Twelve replicates were used for each concentration and the plants were harvested 42 days after sowing.

Flow-through system analysis

At 3 and 10 days after the start of O_2 exposure, the total root respiration (TTR) of intact sealed roots systems of 3 replicates per O_2 concentration was measured using an IRGA (InfraRed Gas Analyser; ADC, UK). Nitrogen fixation was measured as ethylene production using a Perkin Elmer 8310 Gas Chromatograph, equipped with a hydrogen flame ionization detector with a column filled with Porapak R, using nitrogen as a carrier gas at a flow rate of $50 mL \cdot min^{-1}$. These measurements were made using an open flow-through gas system (Minchin et al. 1992) located in the greenhouse (Fernández-Pascual

Table 1. Growth parameters per plant and protein content (mg·g⁻¹ nodule) of nodules of *L. albus* L. cv. Multolupa plants grown under 1%, 21%, and 40% (v/v) O₂ for 3 and 10 days. Data are means (n>3). Values denoted by the same letter within each row do not significantly differ at P < 0.05 (t-student test).

	3 Days			10 Days		
	1% O ₂	21% O ₂	40% O ₂	1% O ₂	21% O ₂	40% O ₂
Shoots						
FW (g)	14.72 a	14.83 a	15.02 a	12.41 b	13.67 a	13.91 a
Roots						
DW (g)	1.00 a	0.88 b	1.03 a	0.55 b	0.95 a	0.99 a
Nodules						
DW (g)	0.12 b	0.11 c	0.13 a	0.09 c	0.11 b	0.17 a
area (m ²)	2.3 10 ⁻³ b	1.5 10 ⁻³ c	2.5 10 ⁻³ a	1.4 10 ⁻³ c	2 10 ⁻³ b	2.5 10 ⁻³ a
Plant cytosol						
protein cont. (mg·g ⁻¹ nodule)	20.14 ab	21.33 a	19.36 b	13.91 b	19.57 a	18.60 a
Bacteroids						
protein cont. (mg·g ⁻¹ nodule)	2.55 b	2.67 b	2.89 a	0.79 c	2.02 b	2.51 a

et al. 1996 b), with a gas stream of 400 mL min⁻¹ plant⁻¹ containing 21% (v/v) O₂. The measurements of respiration and ethylene production started when 10% (v/v) C₂H₂ was added. After the first hour of acetylene application, the O₂ concentration of the gas stream was increased in 10% steps every 30 minutes, up to 80%.

The values of growth and maintenance respiration (GMR) and nitrogenase linked respiration (NLR) (Minchin et al. 1989) were obtained by linear regression of the respiration and C₂H₄ production data. This was used to calculate the total flow of oxygen across the diffusion barrier (F) assuming an RQ of 1 (Minchin and Witty 1990). Total diffusion resistance (R) was calculated as $R = O_e/F$ (O_e = external O₂ concentration) using a modified equation for Fick's first law of diffusion, with the assumption that O₂ concentration of the bacteroids microenvironment was equal to zero. Components of the oxygen diffusion resistance, R_{min} (R calculated for a value of O_e of zero) and RA_r (representing a resistance adjustment factor, c), were calculated by the exponential method ($R = R_{min} \cdot e^{cO_e}$) of Minchin et al. (1992). Volume of known numbers of nodules of each plant was estimated by displacement of water to calculate individual nodule area, assuming that these nodules had the same size and were all spherical (Minchin et al. 1989).

Western blot analysis

Polyclonal antibodies raised against nitrogenase components I and II were used for Western-blot analysis. The component I antibody from the nitrogenase of *Klebsiella* was donated by Dr. Imperial (CSIC & ETSI Agrónomos, Universidad Politécnica de Madrid, Spain), and the component II universal antibody was obtained from Dr. Ludden (Department of Biochemistry, University of Wisconsin, U.S). An antirabbit IgGs conjugated with alkaline phosphatase was used as a secondary antibody. Nodule extracts were prepared as previously described by Fernández-Pascual et al. (1996 a). Nodule pellet was resuspended into Tris-HCl 50 mmol/L pH 5.4, sucrose 1.5 mol/L, DTT 5 mmol/L buffer to obtain bacteroids, spun off for 30 s, and the supernatant centrifuged at 10,000 g for 2 minutes. The pellet containing bacteroids

was washed 6 times with the buffer containing sucrose 0.5 mol/L and resuspended into the buffer containing sucrose 1 mol/L. Bacteroids were disrupted by sonication with 8 pulses of 20 s. Protein content was estimated according to Bradford (1976) using BSA as the standard. SDS-PAGE was carried out under denaturing conditions according to Laemmli (1970), using polyacrilamide 12.5% gels. Electrophoretic analyses were carried out as described by Fernández-Pascual et al. (1996 a).

Results

Growth parameters

General growth parameters of lupin plants were not substantially altered after 3 days of exposure to different oxygen pressures (Table 1). However, root dry weights and nodule parameters increased with both 1 and 40% O₂ exposure for 3 days. The increase in nodule parameters was still present after 10 days of exposure to 40% O₂, but all parameters decreased significantly with respect to the controls when 1% O₂ was applied for 10 days. Bacteroidal protein content diminished dramatically after 10 days of 1% O₂ application, while plant protein content was less affected. In the nodules of the 40% O₂-treated plants, there was a moderate increase in bacteroidal protein content (Table 1).

Nitrogen fixation

After 3 days of the beginning of O₂ treatment, nitrogenase activity of 1% O₂-treated plants was only 25% of the controls (Fig. 1A, Table 2), and this low activity did not increase during O₂ stepping. The plants grown under 40% O₂ also had an inferior nitrogenase activity, but this recovered during oxy-

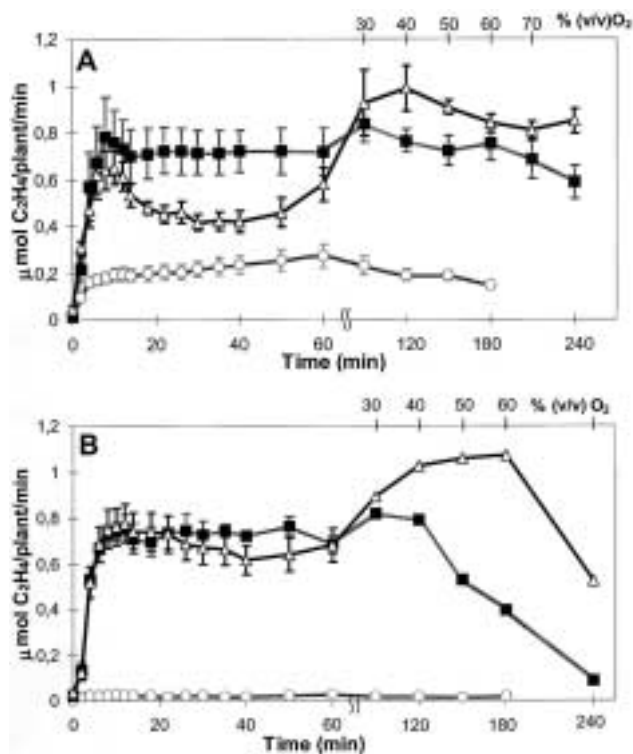


Figure 1. Effect of (A) 3 and (B) 10 days exposure to different concentrations on nitrogenase activity measured as C_2H_2 reduction activity with a flow-through system. (○) 1 % oxygen, (■) Control 21 % oxygen, (△) 40 % oxygen. Numbers on the top of upper line indicate the increase of oxygen concentrations from 21 to 80 % (Oxygen stepping). Data are means \pm SE ($n=3$).

gen stepping and became superior to the control (21 % O_2) plants (Table 2).

The 1 % O_2 application for 10 days completely removed nitrogenase activity; it was not even detectable under increasing O_2 concentrations in the gas stream (Fig. 1B). After 10 days of 40 % O_2 application, the maximum rates of nitrogenase activity did not differ from controls kept at 21 % O_2 . However, the absolute maximum, obtained during O_2 stepping, was higher than the controls and was reached, as at 3 days, at a higher O_2 concentration.

Nodule respiration

After 3 days of growing the plants under 1 % O_2 , there was a substantial reduction in total nodule root respiration (TRR), which was not reversed by a stepped increase in O_2 concentration from 21 % to 80 % (Fig. 2). The values of GMR and NLR obtained from TRR at the beginning of the oxygen stepping (60 minutes after the start point) were both lower than the controls (21 % O_2). The respiration of 40 % O_2 -treated plants was also less than controls in all of the measured parameters. The data for the C costs of nitrogenase, i.e., the

slope of the TRR vs. ARA line, showed a reduction in both treatments (Table 2).

After 10 days of O_2 exposure, the root systems grown at suboptimal oxygen (1 %) again showed a lower respiration rate that was more-or-less constant during the flow-through analysis (Fig. 2). The carbon cost of 1 % O_2 -treated plants was impossible to calculate because nitrogenase activity was zero. The TTR data of the 40 % O_2 -treated plants was also significantly less than the controls. GMR was lower, as happened at 3 days, whilst NLR was higher (Table 2).

Nodule oxygen diffusion resistance

After 3 days of treatment, there was a significant increase in the calculated oxygen diffusion resistance for both 1 and 40 % O_2 plants (Table 2). This was due to an increase in the minimum resistance component (R_{min}), while the variable adjustment component (RA_f) was slightly reduced (Table 2). The calculated resistance data corresponding to the 1 % O_2 treatment was abnormally high, which reflects the low respiration values. Resistance calculations are valid over a range of physiological conditions, but the accuracy may decrease when NLR gets closer to zero. At 1 % O_2 , NLR was reduced by 77 % compared with the control plants. The O_2 diffusion resistance of the plants exposed to 1 % O_2 for 10 days could not be cal-

Table 2. Effect of the different O_2 treatments for 3 and 10 days on nitrogenase activity measured ($\mu\text{mol } C_2H_4 \text{ plant}^{-1} \text{ min}^{-1}$), total root respiration (TRR: $\mu\text{mol } CO_2 \cdot \text{plant}^{-1} \text{ min}^{-1}$), growth and maintenance respiration (GMR: $\mu\text{mol } CO_2 \cdot \text{plant}^{-1} \text{ min}^{-1}$), nitrogenase-linked-respiration (NLR: $\mu\text{mol } CO_2 \cdot \text{plant}^{-1} \text{ min}^{-1}$), and nitrogenase carbon cost ($\mu\text{mol } CO_2 \cdot \mu\text{mol } C_2H_2^{-1}$). Characterization of the total oxygen-diffusion resistance (R) into R_{min} , RA_f was by the exponential method. Values of R are calculated for 21 % O_2 in the presence of acetylene. Data are means ($n = 3$). Values denoted by the same letter within each row do not significantly differ at $P < 0.05$ (t-student test).

	3 Days			10 Days		
	1 % O_2	21 % O_2	40 % O_2	1 % O_2	21 % O_2	40 % O_2
Nitrogenase						
Maximum	0.19 c	0.78 a	0.65 b	–	0.88 a	0.78 a
Max. O_2 step.	0.27 c	0.83 b	0.99 a	–	0.82 b	1.07 a
% O_2 at max.	21	30	40	–	30	60
Respiration						
TRR	3.65 c	7.82 a	4.76 b	1.87 c	6.48 a	5.68 b
NLR	0.43 c	1.86 a	1.52 b	–	2.01 b	2.61 a
GMR	3.21 c	5.99 a	3.24 b	1.87 c	4.46 a	3.07 b
Carbon cost	1.72 c	6.75 a	4.11 b	–	6.51 a	4.45 b
Resistance						
R_{min}	2.00 a	0.12 c	0.53 b	–	0.19 b	0.36 a
RA_f	0.05 b	0.08 a	0.05 b	–	0.04 a	0.02 b
R21	2.67 a	0.26 c	0.85 b	–	0.26 b	0.41 a

culated because nitrogenase activity was totally removed, and therefore, NLR was by definition, zero (Table 2).

After 10 days, the 40 % O₂ diffusion resistance (Table 2) was still significantly higher than the control plants, with an increased R_{\min} and a reduced RA_f . These parameters correspond to a modelled exponential function: R_{\min} is a constant of this function, and corresponds with the calculated value of R when external O₂ concentration (O_e) is zero. In the case of the 40 % O₂ application, this parameter increased by more than 4-fold after 3 days and was still nearly double that of the control plants after 10 days. The reduction in RA_f was much smaller. This adjustment factor represents the adaptation of O₂ diffusion resistance to an increase of O_e . Figure 3 represents the effect of the O₂ treatments on the oxygen diffusion resistance. The two curves are almost parallels (it is sharper in the case of 10 days), and the gap between each pair of values reflects graphically the more intense variation in R_{\min} than RA_f (Fig. 3).

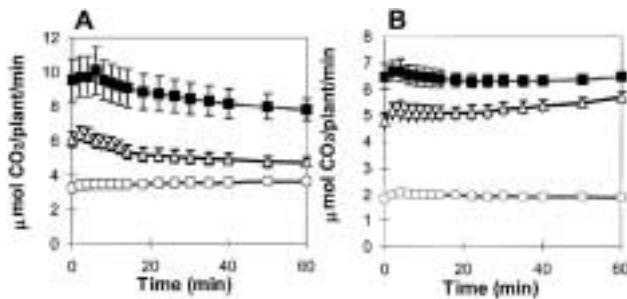


Figure 2. Effect of the oxygen treatments for 10 days on nodule respiration (CO₂ evolution) measured with an IRGA coupled to a flow-through system. **A)** CO₂ production versus oxygen concentration after 3 days of oxygen application. **B)** Rate of CO₂ production after 10 days treatment (Oxygen stepping). (○) 1% oxygen, (■) Control 21% oxygen, (Δ) 40% oxygen. Data are means ± SE (n=3).

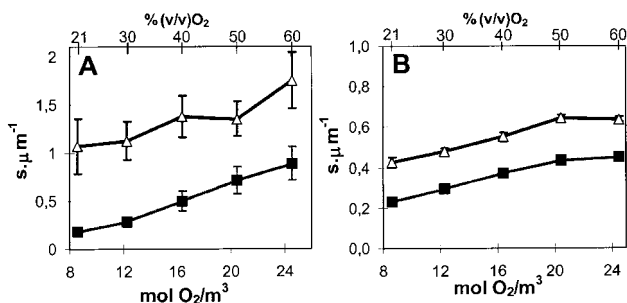


Figure 3. Effect of the different oxygen treatments on the calculated oxygen diffusion resistance. **A)** Oxygen diffusion resistance after 3 days of treatment. **B)** Oxygen diffusion resistance after 10 days of treatment. Numbers on the top upper line indicate the increase of oxygen concentrations from 21 to 80%. (■) Control 21% oxygen, (Δ) 40% oxygen. Data are means ± SE (n=3).

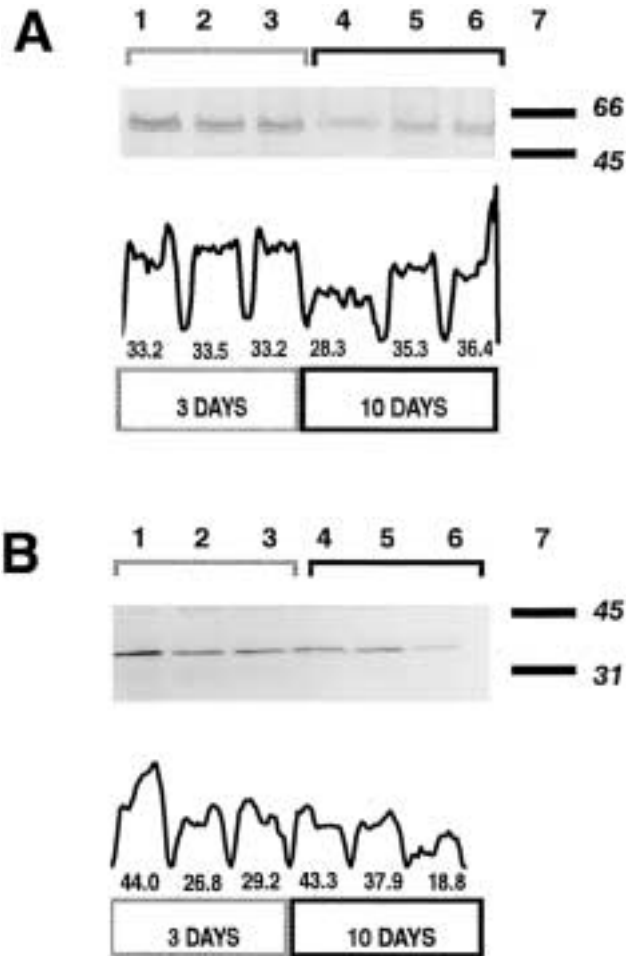


Figure 4. **A)** Nitrogenase component I identified by Western-blotting using nitro-cellulose membranes and densitogram of its relative content for each treatment. 35 μg of bacteroidal protein were loaded to each lane. **B)** Nitrogenase component II identified by Western-blotting and densitogram of its relative content. 5 μg of bacteroidal protein were loaded to each lane. Lanes 1–3: 1%, 21%, 40% O₂, 3 days, lanes 4–6: 1%, 21%, 40% O₂ 10 days, lane 7: low molecular weight markers in kDa. Bovine serum bovine 66, ovalbumin 45, carbonic anhydrase 31.

Western blot analyses of nitrogenase

The nitrogenase component I (MoFe protein) content did not change during 3 days exposure to 1 or 40 % O₂, but a 20 % decrease was observed after 10 days of 1 % O₂ application (Fig. 4). Additionally, there was a reduction in bacteroidal soluble protein (Table 1), so the actual reduction was even higher. Component II of nitrogenase (Fe protein), which is more sensitive to an excess of O₂, showed no reduction for 1 % O₂ plants compared to the controls, whilst there was a significant reduction for 40 % O₂ plants after 10 days (Fig. 4).

Discussion

Oxygen treatments produced important alterations in plant growth. In the case of 1 % O₂, plants were not able to adapt and so nitrogen fixation and growth parameters diminished. In contrast, nodules grown under 40 % O₂ for ten days exhibited adaptations, allowing normal plant growth.

After 3 days of treatment, nitrogenase activity of the 1 % O₂-treated plants could only recover to 30 % of the control plant activity, although the content of the two nitrogenase components was equivalent. After 10 days, the reduction of component I coincided with the total and unrecoverable loss of nitrogenase activity. The bigger reduction in bacteroidal protein content intensifies the changes detected by the Western blots; however, the loss of nitrogenase activity cannot be explained only by these changes. The effects on nitrogenase were not enough to destroy all the epitopes recognised by the antibodies, but were sufficient to eliminate the activity. Also, the lack of relationship between nitrogenase activity and the detection of nitrogenase components has been reported in bacteroids from ineffective nodules of pea, but containing components I and II (Suganuma et al. 1998). It can be argued that nitrogenase components detected from 1 % O₂ nodules were synthesised before, but component II half-life is supposed to be only two days (Bisseling et al. 1980).

The lack of activity produced by the lower O₂ concentration can be related to an O₂ limitation to the bacteroids. Supporting this statement, previous short-term experiments (1 day) showed that 1 % and 21 % O₂-treated plants completely lost nitrogenase activity, at 1 % O₂, and it was totally recovered by turning back to 21 % O₂, without any variation in nitrogenase components (Guasch 1998). Kuzma et al. (1999), exposing the nodules to subambiental O₂ levels, only found variations in the adenylate pools of an enriched bacteroidal fraction, so they assumed that O₂ limitation would be mainly in the bacteroid environment. However, a putative limitation of mitochondrial respiration in the nodule has been postulated (Bergersen 1997b, Appleby 1994), and presumably that limitation should be stronger under hypoxic conditions. In fact, mitochondrial cytochrome oxidase K_m is 50 nmol/L and the terminal oxidase of bacteroids has a K_s of 7 nmol/L (Millar et al. 1995), so the mitochondrial one should be the most affected.

The reduction of TRR with the 1 % O₂ treatment was predictable, and consequently, NLR. Growth and maintenance respiration were also reduced, which may reflect a reduction in metabolic activity. NLR does not refer only to respiration that is directly related to nitrogenase activity. It also relates to all the respiration of nodule components that are inside the barrier. Thus, the respiration of infected cell mitochondria will be included in NLR but not GMR. However, if GMR is reduced, root respiration and nodule outer cortex respiration may also be affected. The dramatic reduction of the available O₂ produced by the 1 % O₂ treatment also reduced GMR. So, in this particular case, root mitochondria and infected cell mitochondria would both be affected, and this effect would

be partially included in GMR. The carbon cost of nitrogenase for 1 % O₂ nodules had a low value that may be affected by severe metabolic changes that were finally reflected in minor plant growth.

Regarding 40 % O₂ treatment, after 3 days there was only a decrease in nitrogenase activity. However, this was not translated into nitrogenase components alterations and it was recovered by increasing oxygen concentration. In spite of the reduction in component II (the most sensitive to an excess of O₂) after 10 days of application, nitrogenase activity at this stage had returned to the level of the control values. Therefore, an additional physiological regulation affects the functioning of nitrogenase, independent of the alteration of its components.

A noticeable effect of 40 % O₂ was the reduction in respiratory activity from 3 days of treatment and the corresponding increase in the calculated O₂ diffusion resistance, despite the presumed effects on mitochondrial and bacteroidal respiration increasing external O₂ (Bergersen 1996, 1997b). The collapse of Lb-facilitated O₂ diffusion has been proposed as the main mechanism of O₂ control in infected cells (Thumfort et al. 1994). If this collapse has reduced nodule respiration, as the suction force, O₂ gradient would be reduced and be translated into an increase of O₂ diffusion resistance. As an alternative, the increase in resistance could have overcompensated for the increase to 40 % O₂, resulting in a decrease in TTR and nitrogenase activity.

Bradyrhizobium cannot uncouple respiration from ATP; therefore, an excess of energy can only be translated into growth or into reserve products such as 3-β-polyhydroxybutyrate (PHB) (Day and Copeland 1991). Actually, there was an increase in bacteroidal protein content at 40 % O₂, and the bacteroids observed by TEM showed a high increase of PHB grains (Guasch 1998). PHB represents a store of acetyl-CoA produced when a substrate is oxidized under micro-aerobic conditions in excess of the capacity of the respiratory chain (Day and Copeland 1991). The increase of bacteroidal protein and the presence of these PHB grains, despite the reduction in respiration, could represent evidence of a new equilibrium of energetic balance caused by a decrease in nitrogenase function producing a decreased sink for the respiratory chain products. In contrast to *Rhizobium meliloti* strains, *Bradyrhizobium japonicum* bacteroids are able to accumulate PHB and fix nitrogen simultaneously (Cevallos et al. 1996), so a certain increase of the microaerobic oxygen concentration could represent a more efficient nitrogenase function (in fact carbon cost is reduced), and the excess energy could be translated into PHB and bacteroidal growth.

In conclusion, the unrecoverable loss of nitrogenase (75 %) after only 3 days of growth under 1 % O₂ shows that the oxygen concentration inside the nodule is a very limiting factor for nitrogen fixation, and that the nitrogenase system can be very sensitive to hypoxic conditions. So the decrease on nitrogenase activity produced by nitrate could be caused simply by the reduction of the oxygen available into the in-

ected zone. The progressive adaptation of 40 % O₂-treated plants to this concentration was obtained through the development of a sustainable higher resistance (R_{\min}) to external O₂ concentration, maintaining a similar capability of response to a stepped increase of O₂ concentration as the control plants (R_{air}). In both treatments, the nitrogenase inhibition was caused more by physiological regulation than by the destruction of the enzyme. Finally, it seems that there are several interactive oxygen regulation pathways: the O₂ diffusion barrier, the long-term morphological alterations (Guasch 1998), and the collapse of Lb-facilitated O₂ diffusion could be considered as a part of the interaction between O₂ regulation and nitrogen fixation in which the relation between nitrogenase activity and PHB synthesis could be involved.

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